Identification of Serine-143 as the Most Likely Precursor of Dehydroalanine in the Active Site of Histidine Ammonia-lyase. A Study of the Overexpressed Enzyme by Site-Directed Mutagenesis†

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ABSTRACT: The gene coding for histidase (histidine ammonia-lyase, HAL, EC 4.3.1.3) was isolated from a λ-EMBL3 genomic library from *Pseudomonas putida* nicII and subcloned into the expression vector pT7-7. Transformation of *Escherichia coli* BL21(DE3) cells with the recombinant vector led to the expression of catalytically active histidase amounting to 20–30% of the total soluble protein in the crude cell extract. A new rapid and highly efficient isolation procedure is described leading to electrophoretically homogeneous histidase within 1.5 days. Six grams of *E. coli* BL21(DE3) cells (wet weight) gives approximately 100 mg of homogeneous histidase with a specific activity of 27 IU/mg. To investigate the possible role of serine as a precursor of dehydroalanine in the active site of histidase, each of the four serines, conserved in all known histidases and phenylalanine ammonia-lyases, was consecutively changed to alanine by site-directed mutagenesis. The resulting mutant genes were subcloned into the expression vector pT7-7 and were assayed for histidase activity. The catalytic activities of the four mutants and of wild-type histidase were compared. The K_m and V_{max} values of the overexpressed mutants S112A, S393A, and S418A and wild-type histidase did not show any significant differences. Mutant S143A, however, was devoid of catalytic activity (<0.01%), pointing to the outstanding importance of this serine for the formation of an active enzyme. We conclude that serine-143 is the most probable precursor of the active-site dehydroalanine. The role of serine-143 in the biosynthesis of active histidase is discussed. To confirm the correct folding of the essential mutant S143A, CD spectra of active wild-type histidase and of mutant S143A were compared. The resulting θ values showed no differences, indicating similar or identical three-dimensional structures. The α-helix:β-sheet:coil plus turn ratio was calculated to be 78%:0%:22%.

Histidase (histidine ammonia-lyase, HAL, EC 4.3.1.3) is the first enzyme in the metabolism of histidine which is finally degraded to L-glutamate and ammonia [reviewed by Hanson and Havir (1972)]. The enzyme catalyzes a nonoxidative elimination of the amino group, leading to the formation of urocanate and ammonia.

For about 40 years, research on histidase has focused on the nature of the active site. Tabor and Mehler (1955) reported the inactivation of histidase by KCN. Smith et al. (1967) showed that other nucleophiles like phenylhydrazine, $NaHSO_3$, and $NaBH_4$ also inactivate the enzyme. Treatment with NaB^3H_4 led to incorporation of tritium into the protein, concomitant with inactivation. Wickner (1969) located the tritium in alanine and concluded that dehydroalanine is present in histidase. This was also found by Givot et al. (1969), who showed that nitromethane reacts with histidase and gives rise to aspartate after total hydrolysis of the protein.

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5), which catalyzes a similar elimination by converting phenylalanine into cinnamate and ammonia, has also been found to contain dehydroalanine (Hanson & Havir, 1970).

In the next decades, several investigators tried to elucidate the origin of the active-site dehydroalanine. For PAL, Hanson and Havir (1981) postulated cysteine or serine as possible precursors. Consevage and Phillips (1985) treated histidase with $K^{14}CN$ and found, after total hydrolysis of the modified protein, [^{14}C]aspartate. To probe serine as the precursor of dehydroalanine, they fed [U-^{14}C]serine to *Pseudomonas putida* (ATCC 12633) and isolated the radioactive histidase. Treatment thereof with *unlabeled* KCN followed by total hydrolysis and 2D chromatography of the amino acid mixture revealed aspartate not to have "appreciable radioactivity above that found in other unrelated amino acids" whereas "serine plus glycine were labeled to a large degree". They concluded that this result seemed to rule out serine as a precursor of dehydroalanine.

We did not believe that this conclusion is justified (the reasons for it are under Discussion) and undertook a different approach to solve the problem. Here we report our experiments that led us to conclusions varying from those of Consevage and Phillips (1985).

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions. E. coli SURE cells [restriction-negative mcrA, Δ(mcrCB-hsdSMR-mrr)^{171}, and endonuclease-deficient; purchased from Stratagene] were used for the isolation of single-stranded (ss) DNA from M13 phages to carry out site-directed mutagenesis. Cells were grown and infected as described in the lab manual (Sambrook et al., 1989).

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E. coli BL21(DE3) cells served for the expression of either wild-type or mutant histidase. For overexpression, cells were grown at least 16 h in 1 L of Luria–Bertani medium supplemented with ampicillin (85 μg/mL) at 37 °C without induction with isopropyl thio-β-D-galactoside.

The expression vector pT7-7 was generously donated by Dr. Stanley Tabor (Tabor & Richardson, 1985). pT7-7H was obtained by subcloning the hut H gene into pT7-7 by using the restriction sites *Nde*I at the 5′ end of the gene (created by the use of site-directed mutagenesis) and *Eco*RI at the 3′ end as described under Subcloning.

The phages M13mp18 and M13mp19 were from Boehringer Mannheim. pBR322 was purchased from New England Biolabs and pGEM 11-Zf-(-) from Promega.

Subcloning the hut H Gene. The hut H gene was isolated as described by Lenz and Rétey (1993) from a λ-EMBL3 (European Molecular Biology Laboratory) clone obtained from a genomic library of *P. putida* nicII (Fessenmaier et al. 1991). A *Bam*HI and *Eco*RI digest resulted in several fragments. A 6.4 kbp fragment, containing the hut H gene downstream of the hut U gene, was isolated by electrophoresis and subcloned into pBR322. A *Pst*I digest of the recombinant plasmid led to a 700 bp fragment which included the start codon of the hut H gene. This fragment was cloned into the phage M13mp18 for the introduction of a 5-terminal *Nde*I site. Digestion with *Nde*I and *Pst*I of recombinant M13mp18 resulted in a fragment coding for the first 77 amino acids of histidase. This fragment was subcloned in pT7-7 which contained the 3′ end, a *Pst*I–*Eco*RI, fragment from recombinant pBR322, coding for the missing 433 amino acids. A complete list of the strains and vectors used in this study is given in Table 1.

Site-Directed Mutagenesis. Recombinant pT7-7H was used to subclone either a *Xba*I–*Sal*I fragment into M13mp19 or a *Sal*I–*Eco*RI fragment into M13mp18. Starting with these two clones, Ser/Ala exchange was performed. The mutant DNA then was patched up with the missing DNA fragment by subcloning it into pGEM 11-Zf-(-) vector. Each mutant was then inserted into pT7-7 by the use of the 5-terminal *Nde*I site and the 3-terminal *Eco*RI site. Site-directed mutagenesis was performed as described by Sayers et al. (1988) following the protocol of the Amersham mutagenesis kit (version 2.1). The S393A exchange was made by a novel, improved Amersham mutagenesis kit (Sculptor), to avoid secondary structures of the ssDNA used in the reaction.

Oligonucleotides used in mutagenesis reactions were

NdePr: 5′-GGAGTAGTCATATGACCGAACTC-3′

S112A: 5′-CGTGGCTTCGCAGGCATTCGC-3′

S143A: 5′-GTGGGTGCTGCCGGCGACCTG-3′

S393A: 5′-CTCTGGCCGCAGAGAACAAGG-3′

S418A: 5′-GACCACGTAGCGATGGCCCCG-3′

The mutagenesis reaction for the insertion of a *Nde*I site was checked by restriction analysis of double-stranded DNA of the M13mp18 clones. The Ser/Ala exchanges were confirmed by sequence analysis using the dideoxynucleotide chain-termination method of Sanger et al. (1977). Either Sequenase (USB) at 37 °C or Taq-polymerase (Boehringer Mannheim) at 70 °C was used for the sequencing reaction (Innis et al., 1988). In some cases, both enzymes were used for sequencing high G+C% DNA (Khambaty & Ely, 1990).

Table 1: Mutants Used for Histidase Isolation[a]

strain	plasmid	phenotype
P. putida nicII	none	leaky, auxotrophic for nicotinate
pT7-7H	pT7-7 including hut H (gene pT7-7H)	expression of wild-type histidase
S112A	pT7-7H (S112 → A)	S112A mutant
S143A	pT7-7H (S143 → A)	S143A mutant
S393A	pT7-7H (S393 → A)	S393A mutant
S418A	pT7-7H (S418 → A)	S418A mutant

[a] The nomenclature refers to the position of serine/alanine exchange in the recombinant protein. If not otherwise stated, the host cells for the plasmids were *E. coli* BL21(DE3).

Transformation. Either *E. coli* BL21(DE3) or *E. coli* SURE cells were grown in 0.5 L of LB medium to an OD_{600} of 0.3–0.4. The cells were sedimented by 4500*g*. The cell pellet was resuspended in 0.5 L of ice-cold 10% glycerol. The cells were made competent by successive reduction of the resuspension volume of 10% glycerol in five steps. The final volume of the competent cells was 1 mL. All steps were performed at 4 °C; 80-μL aliquots of the concentrated competent cells were stored at −70 °C for 2 months without loss of competence. Transformation was performed by electroporation using a gene pulser from Biorad. Seventy microliters of competent cells was transformed with 20 ng of vector DNA in a 0.4-mm cuvette at 2.5 kV and 0.2 kΩ (6.25 kV/cm) for 4.4 ms. Recombinant phages were selected by the aid of blue/white screening. Recombinant bacteria were detected either by blue/white screening or by restriction site analysis of grown colonies.

Purification. Transformed *E. coli* BL21 cells were grown in 1 L of LB medium containing 85 mg/L ampicillin at least 16 h at 37 °C and harvested by centrifugation at 8000*g* and 4 °C for 10 min. The cell pellet (about 6 g wet weight) was suspended in 12 mL of 20 mM Tris-HCl, pH 7.2, supplemented with 40 units of Benzonase (Merck, Darmstadt), 5 mM benzamidine, and 0.5 mM phenylmethanesulfonyl fluoride. Sonication (Branson Model 450, 70% power setting) in an ice bath was carried out for 10 min, and the crude cell extract was centrifuged at 30000*g* for 30 min. In the first purification step, the clear supernatant was heat-treated in an 80 °C bath for 5–10 min to precipitate most of the soluble proteins. The flocky suspension was centrifuged at 30000*g* for 30 min. The filtered supernatant (0.45-μm filter) was then applied to a HiLoad 26/60 Superdex 200 preparative-grade column and isocratically fractionated at a flow rate of 1.75 mL/min using FPLC equipment (Pharmacia); 100 mM potassium phosphate buffer, pH 7.2, was used as eluent. Histidase eluted after 120 min. Fractions of high activity were pooled, concentrated to a final protein concentration of 1 mg/mL by ultrafiltration, and diluted with water to a final potassium phosphate concentration of 20 mM, pH 7.2, by the use of a stirred cell with a nominal molecular mass limit of 30 kDa.

The final purification of histidase was performed by anion-exchange chromatography with a HiLoad 16/10 Q-Sepharose high-performance column using 20 mM potassium phosphate buffer, pH 7.2, as buffer A and as eluent buffer A plus 1 M NaCl. At a flow rate of 3 mL/min, fractions were taken every 3 min. Histidase was eluted at a concentration of approximately 150 mM NaCl. Pure histidase was then brought to a final concentration of 2 mg/mL and stored at +4 °C.

SDS/PAGE and Protein Assays. SDS/PAGE was performed according to Laemmli (1970) using 10% polyacrylamide gels to monitor the purification of histidase. Staining

of the gels was carried out with Coomassie Brillant Blue R 250. Protein determinations were performed either using the Bradford test with bovine serum albumin as a standard (Bradford, 1976) or by micro biuret measurements (Itzhaki & Gill, 1964) or by measurement of A_{260} and A_{280} according to Warburg and Christian (Lane, 1957).

Enzyme Assay. Histidine ammonia-lyase activity was determined by measuring the rate of formation of urocanate per minute as indicated by increasing absorbancy at 277 nm (Mehler & Tabor, 1953). For evaluation of the kinetic parameters, a standard assay with the indicated parameters was used: 2.5 mL of 0.1 M pyrophosphate buffer, pH 9.3, containing 10 μM $ZnCl_2$ was incubated for 5 min with 50 μL of 0.1 M glutathione and 10 μL of enzyme solution (2 μg of protein in the cuvette) at 25 °C. The reaction was started with 200 μL of 0.5 M histidine solution, giving a final concentration of 36 mM. The measurements were performed at 25 °C and were confirmed 6–8 times. K_m and V_{max} values were calculated by a double-reciprocal plot (Lineweaver & Burk, 1934). The extinction coefficient (ϵ_{277}) of urocanic acid is 18 800 L mol^{-1} cm^{-1}.

Circular Dichroism (CD) Spectra. Far-UV circular dichroism spectra of wild-type and mutant histidase were measured using a Jasco J-500 spectralpolarimeter coupled to a J-DPY digital data processor. The instrument was calibrated using a 0.05% solution of β-androsterone in dioxane. The slit width was automatically maintained at 1.0 nm throughout the spectral range.

Wild-type histidase and the S143A mutant[1] were dialyzed against Tris-HCl, pH 7.2, and diluted to a final concentration of 100 μg/mL. Spectra were measured over the range of 190–240 nm at a sensitivity of 2.0 mdeg/cm and a scanning speed of 5.0 nm/min with a 2.0-s time constant. Curves presented are the product of four signal-averaged measurements with a similarly signal-averaged base line subtracted.

For purposes of secondary structure analysis, the CD curves were plotted as the mean residue ellipticity, $[\theta_m]$, using the equation:

$$[\theta_m] = \frac{[\theta_{obs}]MRW}{10cp}$$

where θ_{obs} is the observed ellipticity in millidegrees, MRW is the mean residue weight of the protein, p = path length in centimeters, and c = protein concentration in milligrams per milliliter.

RESULTS

Expression of Wild-Type Histidase in E. coli BL21(DE3) Cells. It has been previously described that the use of *E. coli* BL21(DE3) cells leads to high expression of recombinant active urocanase (Lenz & Rétey, 1993; Koberstaedt et al., 1992). The expression and analysis of hut genes in *E. coli* cells are favorable, since in contrast to *P. putida* they lack the hut operon. In the beginning, it was not certain whether the posttranslational modification leading to dehydroalanine, and to active histidase, takes place in *E. coli* cells. Fortunately, the use of the pT7-7 system for expression of histidase led to high expression of fully active recombinant protein. Histidase amounted up to 30% of the total protein. These results indicate an autocatalytic activation of the precursor enzyme to active histidase. Supplementary induction with isopropyl thio-β-D-galactoside had no strong effect on the overexpression, so that the cells were grown without the addition of inducer.

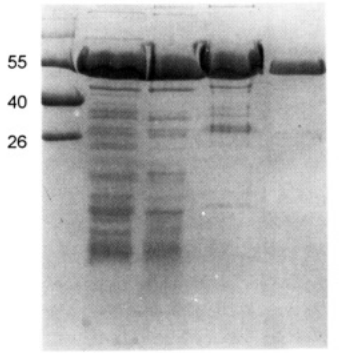

FIGURE 1: SDS/PAGE of recombinant histidase from *E. coli* BL21-(DE3) after each purification step. Lane 1, molecular mass standards; lane 2, crude cell-free extract (30 μg of total protein); lane 3, supernatant after heat treatment (30 μg of total protein); lane 4, pooled protein fraction after FPLC gel filtration (20 μg of total protein); lane 5, pooled purified histidase after FPLC Q-Sepharose column (12 μg of histidase). The molecular masses of the markers are indicated (in kilodaltons).

Purification of the Recombinant Protein. The method described here is a rapid and very effective means to produce large amounts of recombinant histidase. In contrast to the time-consuming purification of histidase starting from *P. putida* (Rechler, 1969), isolation from recombinant *E. coli* leads to an electrophoretically homogeneous preparation within 1.5 days. A routine isolation starting with 1 L of Luria–Bertani medium allowed harvesting of 6 g of wet cells. From these, the novel purification procedure yields 70–100 mg of homogeneous histidase. SDS–PAGE analysis of the proteins after each purification step is shown in Figure 1.

The elution profile from the Q-Sepharose column is shown in Figure 2. Elution of the protein with 1 M NaCl leads to four active peaks where the first peak with a retention time of 38.8 min represents reduced active histidase with the highest specific activity observed. The second peak at 44.9 min represents a reduced histidase lacking Mg^{2+} ions, as can be proved by eluting the proteins with buffers A and B containing 2 mM $MgSO_4$ (Higashi, 1969), when peak 2 disappears and peak 1 increases. Klee (1972) describes a dependency of histidase from divalent metal cations such as 0.1 mM Mn^{2+} and 0.1 mM Cd^{2+}. Rechler (1969) eluted histidase with buffers containing 0.1 mM Mn^{2+} ions "to prevent loss of activity". Peaks 3 and 4 at 51.3 min and the shoulder of the peak 62.98 min represent oxidized histidase with and without Mg^{2+} ions, exhibiting less specific activity compared to the reduced enzyme (peak 1). Elution of histidase with 2 mM $MgSO_4$ and 1.8 mM glutathione (Tabor & Mehler, 1955) in buffers A and B resulted in a total decrease of peak 4 (shoulder). Peak 3 increased a little, indicating that the oxidation of histidase was not significantly reversed by glutathione, which is in agreement with previous results (Frankfater & Fridovich, 1970).

Table 2 gives a summary of the individual purification steps for recombinant histidase isolated from *E. coli* BL21(DE3).

Comparison of the Expression and the Kinetic Parameters for Wild-Type Histidase and Ser/Ala Mutants. The kinetic parameters K_m and V_{max} of recombinant wild-type histidase were estimated and compared to histidase isolated from *Pseudomonas* ATCC 11299b (Klee et al., 1975) and histidase from *P. putida* (Consevage & Phillips, 1985). The proteins exhibited nearly identical kinetic behavior even after different expression and purification procedures, indicating that over-

[1] Because of the posttranslational removal of the N-terminal amino acid in histidase (Consevage & Phillips, 1990), serine-143 corresponds to serine-144 of the deduced amino acid sequence.

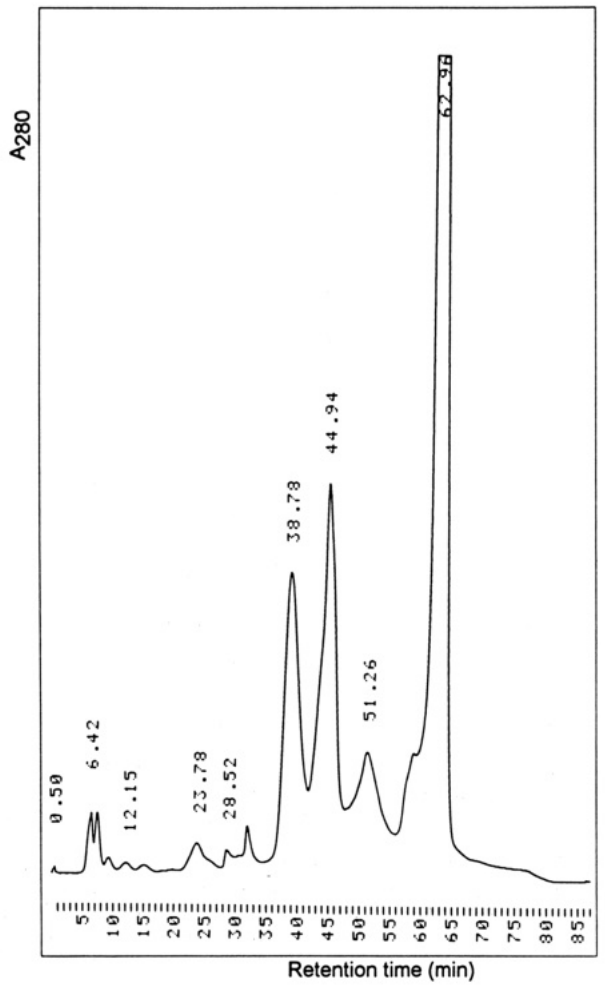

FIGURE 2: Elution diagram for the HiLoad 10/16 Q-Sepharose column of the pooled histidase peak after gel filtration. Histidase was eluted from the column as described under Materials and Methods. The peak at retention time 38.8 min represents reduced histidase which was used for kinetic measurements.

Table 2: Summary of Individual Purification Steps for Recombinant Histidase Isolated from *E. coli* BL21(DE3)[a]

purification step	total protein (mg)	total act. (units)	sp act. (units/mg)	yield (%)
crude extract	460	3270	7.1	100
heat treatment	270	3150	11.7	96
Superdex G-200	157	2850	18.2	87
Q-Sepharose	97	2570	26.5	79

[a] The cells were grown overnight at 37 °C in 1 L of Luria–Bertani medium as described under Materials and Methods.

expression of histidase in *E. coli* leads to a fully active enzyme. For histidase isolated from *P. putida*, the K_m value was estimated to be 5.3 mM with a V_{max} of 25 IU/mg of histidase compared to a K_m value of 3.59 mM and a V_{max} of 25 IU/mg of histidase of recombinant protein and a K_m value of 4 mM and a V_{max} of 28 IU/mg of histidase from *Pseudomonas* ATCC 11299b. The values were obtained at a pH of 9.0 at 25 °C with L-histidine as a substrate as described under Materials and Methods. These results suggest that there is no difference between the synthesis of the protein in the three different bacteria. All four mutant species and the recombinant wild-type enzyme were stable at 4 °C for months. The kinetic behavior from three of the four mutants and that from the recombinant wild-type enzyme were nearly identical. Only mutant S143A showed nearly no activity at all (less than 0.01% of that of the wild-type enzyme), suggesting that this

Table 3: Summary of Kinetic Parameters of Isolated Enzymes Including Histidase Isolated from *Pseudomonas* ATCC 11299b (Klee et al., 1975) and *P. putida* (Consevage & Phillips, 1985) and Recombinant Histidase Isolated from *E. coli* BL21(DE3)[a]

protein	kinetic constants of enzyme	
	K_m (mM)	V_{max} (IU/mg)
wild-type *Pseudomonas* ATCC 11299b	4.0	28
wild-type *P. putida*	5.3	25
recombinant wild-type *E. coli*	3.59	25
recombinant mutant S112A	4.87	25.5
recombinant mutant S143A	not accessible	less than 0.01%
recombinant mutant S393A	3.57	20.3
recombinant mutant S418A	3.52	21.5

[a] All proteins were purified to homogeneity and tested in a standard assay using the fully active reduced form of histidase stored at 4 °C. The kinetic constants were determined from the double-reciprocal plot.

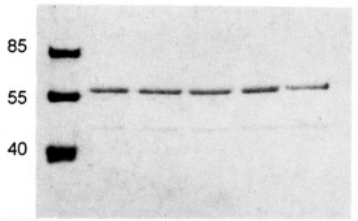

FIGURE 3: SDS/PAGE of recombinant wild-type and mutant histidase. Lane 1, molecular mass standards; lane 2, recombinant wild-type histidase; lane 3, recombinant mutant histidase S112A; lane 4, recombinant mutant histidase S143A; lane 5, recombinant mutant histidase S393A; lane 6, recombinant mutant histidase S413A. Each lane was loaded with 2 μg of protein. The molecular masses of the markers are indicated (in kilodaltons).

serine residue plays a very important role in the catalytic activity of histidase. The K_m and V_{max} values estimated for recombinant wild-type and mutant histidases are summarized in Table 3.

In Figure 3, the SDS/PAGE gel of the purified recombinant histidase and the four mutants is shown. It can be seen that the enzymes used in the kinetic measurements were highly pure, and more importantly, mutant S143A also showed a band at 55 kDa which differs neither in quantity nor in mass from wild-type histidase and from all other mutants.

CD Spectroscopy of Recombinant Wild-Type and Mutant S143A Histidase. Circular dichroism (CD), the property of differentially absorbing right and left circularly polarized light, functions as an extremely sensitive indicator of molecular conformation. In proteins, the amplitude, sign, and to a certain extent position of circular dichroism associated with absorption due to peptide bond transitions are dependent on the θ and Ψ angles of the bond. This makes it possible to estimate the amount and type of secondary structure present in a protein directly from its CD spectrum. A comparison of recombinant active wild-type histidase with the inactive S143A mutant has been made to exclude that improper folding of the S143A mutant is responsible for the lack of catalytic activity. Two CD spectra were taken from 190 to 240 nm: one with recombinant wild-type histidase and the other with the inactive mutant. An overlay plot is shown in Figure 4. The two spectra are identical, indicating that the inactivity of the mutant is not due to any change in secondary structure but is due to the change of serine-143 into a catalytically inert alanine. The curves reveal that in histidase α-helical structures dominate. Analysis by the CONTIN program of Provencher and Glöckner (1981) gives a secondary structure content of 78% α-helix, 0% β-sheet, and 22% coil plus turn.

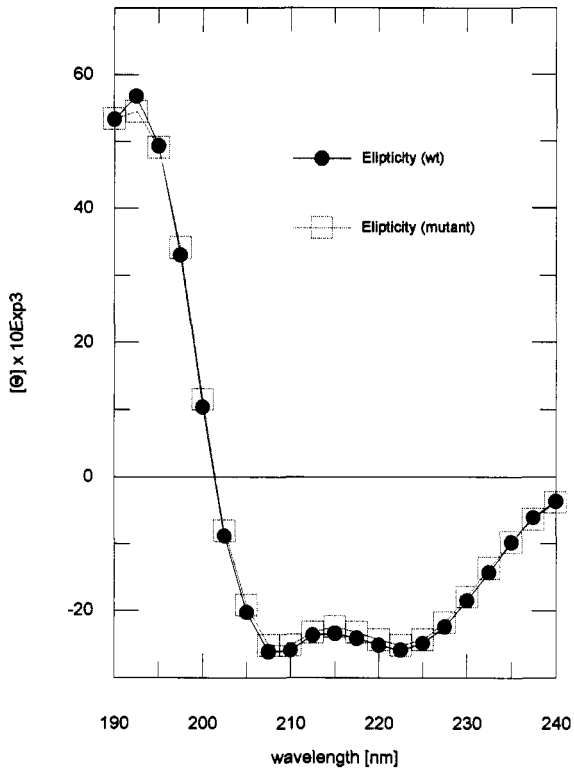

FIGURE 4: Comparison of the far-UV CD spectrum of wild-type and S143A histidase. (●) indicates wild-type protein; (□) indicates S143A mutant. Measurements were preformed as described under Materials and Methods.

DISCUSSION

In the deduced amino acid sequence of histidases from *P. putida* (Consevage & Phillips, 1990), *Bacillus subtilis* (Oda et al., 1988), and *Streptomyces griseus* (Wu et al., 1992) from rat liver (Taylor et al., 1990) and mouse liver (Taylor et al., 1993) and the amino acid sequences of phenylalanine ammonia-lyases from *Rhodotorula toruloides* (Anson et al., 1987), *Rhodotorula rubra* (Filpula et al., 1988), *Oryza sativa* (Minami et al., 1989), *Petroselinum crispum* (Schulz et al., 1989; Lois et al., 1989), *Glycine max* (Frank & Vodkin, 1991) *Ipomea batatas* (Tanaka et al., 1989), *Lycopersicon esculentum* (Lee et al., 1992), and *Phaeseolus vulgaris* (Cramer et al., 1989), four conserved serines were identified. A search for conserved cysteines in the above sequences led to negative results. Therefore, a systematic replacement of each of these serines by alanines was undertaken to identify the precursor of the dehydroalanine at the active site of histidase.

Heterologous expression of histidase was a prerequisite for site-directed mutagenesis. The use of the expression vector pT7-7 led to very successful overexpression that allows isolation of up to 100 mg of electrophoretically homogeneous fully active histidase from 1 L of *E. coli* culture (6 g of wet cells) within 1.5 days. Since *E. coli* does not normally express histidase, the posttranslational modification must occur by a specific autocatalytic process. It will be challenging to identify the domain of histidase responsible for the biosynthesis of the unique, catalytically essential dehydroalanine.

While our manuscript was being prepared, Hernandez and Phillips (1993) published the expression of histidase using another vector, namely, pPL-lambda. In their system, 120 g wet cell mass yielded after purification 870 mg of histidase with a specific activity of 16.7 IU/mg.

Opinions on the origin of the active-site dehydroalanine were controversial. Hassal and Soutar (1974) proposed that it is formed as a consequence of desulfurization of cystine. On the other hand, it is known that dehydration of serine can occur in polypeptides both naturally and artificially [Banerjee & Hansen, 1988; Buchman et al., 1988; for a review, see Jung (1991a,b)].

HAL *P.putida*	138-149	GSVGA**SGDLAPL**
HAL *Str.griseus*	142-153	GSLGC**SGDLAPL**
HAL *B.subtilis*	137-148	GSLGA**SGDLAPL**
HAL *R.norvegicus*	250-261	GTVGA**SGDLAPL**
HAL *M.musculus*	250-261	GTVGA**SGDLAPL**
PAL *R.toruloides*	205-216	GTISA**SGDLSPL**
PAL *R.rubra*	211-222	GTISA**SGDLSPL**
PAL *O.sativa*	184-195	GTITA**SGDLVPL**
PAL *P.crispum*	197-208	GTITA**SGDLVPL**
PAL *G.max*	194-205	GTITA**SGDLVPL**
PAL *I.batatas*	187-198	GTITA**SGDLVPL**
PAL *L.esculentum*	204-215	GTITA**SGDLVPL**
PAL *Ph.vulgaris*	193-204	GTITA**SGDLVPL**

FIGURE 5: Comparison of the amino acid composition of five histidases (HAL) and eight phenylalanine-ammonia lyases (PAL) around serine-143 in histidase from *P. putida*. Comparison was carried out using the ClustAIV program in HUSAR (Heidelberg Unix Sequence Analysis Resources). Strongly conserved invariant residues are marked in boldface letters. Numbers indicate the situation of the highly conserved region in the sequences compared in this alignment. It can be seen that serine-143 is situated in a highly conserved region.

Both Klee and Gladner (1972) and Hassal and Soutar (1974) reported the labeling of histidase from two different strains with iodo[^{14}C]acetate. Tryptic digestion gave a 17-residue radioactive peptide carrying [^{14}C](carboxymethyl)-cysteine. The amino acid composition of the two peptides was very similar.

Consevage and Phillips (1985) labeled histidase with K-^{14}CN. After tryptic digestion, the "major radioactive peptide" had the following amino acid composition: Ala_6, Asp_1, Glx_4, Gly_2, Leu_5, Lys_1, Pro_1, Ser_1, Thr_2, Trp_1, Val_1. It was assumed that the unique aspartic acid arose from the hydrolysis of [^{14}C]-β-cyanoalanine, though the individual amino acids were not analyzed for radioactivity. Closer examination of the deduced histidase sequence published later (Consevage & Phillips, 1990) permits identification of a unique sequence of 25 amino acids (positions 166–190 or 167–191) consistent with the above amino acid composition except that instead of the only Asp a second Lys or a third Gly is present in the deduced sequence. Since none of these amino acids is a likely precursor of dehydroalanine, we conclude that the latter must be elsewhere. Consevage and Phillips (1990) do not discuss the position of this "major radioactive peptide" in the deduced histidase sequence and the possible location of the unique dehydroalanine therein.

In an attempt to test serine as a precursor of dehydroalanine, Consevage and Phillips (1985) labeled histidase with [U-^{14}C]-serine by feeding *P. putida* with this radioactive amino acid. After treatment with unlabeled KCN followed by total hydrolysis and 2D chromatography, they did not find significant radioactivity in the aspartic acid spot relative to the radioactivity of serine and glycine.

Their conclusion that serine is to be excluded as the precursor of dehydroalanine is not tenable, however, since histidase contains 41 Asx and 39 Ser per subunit, (Consevage & Phillips, 1990) so that their aspartic acid could contain at most 2.5% of the radioactivity that was measured in serine. Although no exact data are given, the precision of the radioactivity determinations can be estimated from their Table 2 (Consevage

[2] After submitting our manuscript, we learned about a paper of Hernandez et al. (1993), who modified histidase with cysteine in the presence of oxygen and determined the site of modification to be serine-143.

& Phillips, 1985), in which the results of the complementary experiment ($K^{14}CN$, unlabeled histidase) are summarized. Several unrelated amino acids exhibit 2–9% of the radioactivity of Asp. In the present work, of the four conserved serines only serine-143 was found to be essential for catalytic activity, which makes it a likely precursor of dehydroalanine.[2]

A further indication of the importance of serine-143 is its situation in the sequence Gly-Ser-Val-Gly-Ala-Ser-Gly-Asp-Leu-Ala-Pro-Leu that is highly conserved in all histidase and PAL sequences known so far. A comparison of the amino acid composition close to serine-143 of these sequences is shown in Figure 5. Although at present we cannot exclude that serine-143 plays some other role in the catalytic process, our results strongly suggest it being the precursor of the active-site dehydroalanine. This is supported also by our circular dichroism results which indicate that the inactive S143A mutant has a very similar 3D structure as fully active wild-type histidase.

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